Molecular Screening for GS2 Lipase Regulators: Inhibition of Keratinocyte Retinylester Hydrolysis by TIP47

Jay G. Gao[1] and Marcia Simon[1]

Retinoic acid at nanomolar concentrations modulates epidermal functions by serving as a transcription factor ligand. Under conditions of retinol sufficiency, it is imperative to limit retinoic acid biosynthesis from serum-derived retinol. In the epidermis, this is accomplished by esterifying retinol with long-chain fatty acids. Retinylester (RE) pools serve as a source of retinol for retinoic acid production under retinol deficiency and when required for proper differentiation. We have recently reported that GS2 lipase is expressed in keratinocytes and has the enzymatic properties of keratinocyte RE hydrolase. As GS2 lipase has a robust activity that can affect the intracellular retinol levels, we postulated that its activity must be regulated. Therefore, we screened keratinocyte cDNA expression libraries for the putative inhibitor. Herein, we report the identity of an inhibitor, TIP47, which prevents RE hydrolysis catalyzed by GS2 lipase and hormone-sensitive lipase. This protein was known to transport mannose-6-phosphate receptors from endosome to *trans*-Golgi and to be distributed between the cytoplasm and lipid droplets. Using a series of deletion mutants, we found two regions involved in the inhibitory activity. Residues within the carboxyl α3–α4 helices are essential in the context of the full-length protein. Residues within the amino-terminal also contribute depending on the context.

Journal of Investigative Dermatology (2006) **126,** 2087–2095. doi:10.1038/sj.jid.5700327; published online 1 June 2006

INTRODUCTION

All-*trans* retinoic acid (atRA) has been shown to regulate epithelial differentiation, proliferation, and apoptosis by binding to cognate homo- and heterodimeric nuclear receptors and altering the association of co-activator or co-repressor complexes over retinoid-responsive promoters (Bastien and Rochette-Egly, 2004; Mangelsdorf and Evans, 1995). In the epidermis, atRA can be supplied from the circulation or biosynthesized from serum-derived retinol through a two-step dehydrogenation process (Napoli, 1986; Siegenthaler, 1990; Siegenthaler *et al.*, 1990). The availability of serum atRA is limited by its metabolism in dermal fibroblasts, whereas its biosynthesis from serum-derived retinol is limited by retinol esterification with long-chain fatty acids (Jurukovski and Simon, 1999; Randolph and Simon, 1993, 1998; Napoli *et al.*, 1995; Kurlandsky *et al.*, 1996). Retinol esterification can be catalyzed by lecithin retinol acyl transferase or by acyl coenzyme A retinol acyl transferase (Torma and Vahlquist, 1987, 1990). The contribution of each enzyme is linked not only to their expression but also to the relative concentrations of free retinol and cellular retinol-binding protein (Napoli *et al.*, 1995). Lecithin retinol acyl transferase, which uses phosphatidylcholine as acyl donor, has a low K_m for retinol, and accepts both free and cellular retinol-binding protein-bound retinol as substrate (Shingleton *et al.*, 1989; Randolph *et al.*, 1991; Shi *et al.*, 1993). As its expression is positively regulated by atRA, this enzyme provides negative feedback that limits atRA synthesis under atRA sufficiency even at low concentrations of retinol and at low ratios of retinol:cellular retinol-binding protein (Ross, 2003). In contrast, acyl coenzyme A retinol acyl transferase, which employs fatty acyl coenzyme A as acyl donor, has a high K_m for retinol, and accepts only free retinol as substrate. The contribution of acyl coenzyme A retinol acyl transferase to retinylester (RE) formation is therefore limited to conditions where both the free retinol level and the ratio of retinol:cellular retinal-binding protein is high.

The hydrolysis of REs provides tissues with a local source of substrate retinol, which can be converted to atRA. A number of liver and adipocyte enzymes have been shown to hydrolyze RE including two families of cytosolic carboxyl-esterases, secreted bile salt-dependent ester hydrolases, microsomal bile salt-independent ester hydrolases, and hormone-sensitive lipase (Harrison, 1998; Sanghani *et al.*, 2002). Of these enzymes, human keratinocytes express

[1]*Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York, USA*

Correspondence: Dr Marcia Simon, Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York, USA.
E-mail: marsimon@notes.cc.sunysb.edu

*Abbreviations: atRA, all-*trans *retinoic acid; HSL, hormone-sensitive lipase; MPR, mannose-6-phosphate receptor; PBS, phosphate-buffered saline; RE, retinylester*

Received 20 December 2005; revised 8 February 2006; accepted 9 March 2006; published online 1 June 2006

carboxylesterases ES1 and ES2, and a splice variant of hormone-sensitive lipase (HSL) that can catalyze RE hydrolysis (Gao and Simon, 2005). In addition, we have recently shown that in epidermis keratinocyte, RE hydrolysis is catalyzed by GS2 lipase (also called: iPLA$_2\eta$) (Gao and Simon, 2005). GS2 lipase is a 253-amino-acid protein encoded by a previously sequenced gene, GS2 (gene sequence 2), located at Xp22.3 (Lee *et al.*, 1994). Unlike the carboxylesterases and HSL, this enzyme shares characteristics of the RE hydrolase activity of keratinocyte homogenates. It has a neutral pH optimum and is insensitive to the carboxylesterase inhibitor, bis-*p*-nitrophenyl phosphate. The enzyme is a member of the patatin family of acyl hydrolases, which contain a Ser-Asp catalytic dyad (Rydel *et al.*, 2003). Interestingly, GS2 lipase has a robust lipase activity and catalyzes the accumulation of both diacylglycerols and free fatty acids from triolein (Jenkins *et al.*, 2004; Gao and Simon, 2005). As such, this enzyme has been grouped with an expanding family of enzymes that replace HSL as the activity responsible for the first step in triacylglycerol metabolism (Haemmerle *et al.*, 2002; Lake *et al.*, 2005; Zechner *et al.*, 2005).

As GS2 lipase can mobilize both retinol and fatty acid stores and potentially impact retinoid and non-retinoid lipid signaling, we predicted that its activity must be regulated. We explored this possibility using the two keratinocyte cDNA expression libraries used to identify GS2. Herein, we report the identification of an inhibitor that suppresses the RE hydrolase activity of GS2 lipase, as well as other hydrolytic enzymes. This protein originally identified as a placental protein (PP17) was later renamed tail-interacting protein of 47 kDa (TIP47). Its binds the cation-dependent and cation-independent mannose-6-phosphate receptors (MPR) and Rab9 and mediates the transport of the MPRs from endosomes to the *trans*-Golgi (Diaz and Pfeffer, 1998; Carroll et al., 2001; Barbero *et al.*, 2002). It also associates with lipid droplets and can be recruited to the plasma membrane upon lipid loading (Wolins *et al.*, 2001, 2005; Miura *et al.*, 2002; Brasaemle *et al.*, 2004; Robenek *et al.*, 2005a–c). REs may also be found at or near these subcellular locations. The potential colocalization may enhance the ability of TIP47 to inhibit RE hydrolysis.

RESULTS

Assay development

We first determined the smallest amount of pGS2 needed for detection in co-transfections with the cDNA pools. As shown in Figure 1, a linear increase in RE hydrolysis was observed in transfections using 1–5 ng pGS2. Cultures transfected with 1 ng of pGS2 had RE hydrolase activity two-fold over background; this amount of pGS2 is therefore the lowest level usable. In co-transfection with 2 μg of each cDNA pool (4,000 clones/pool), we theoretically achieve a ratio of GS2 lipase:GS2 lipase inhibitor of about 2:1. These amounts of pGS2 and cDNA pools were used for subsequent screenings.

Identification of a cDNA encoding a GS2 lipase inhibitor

Two of the 48 pools (nos. 2 and 53) initially used to screen for the keratinocyte RE hydrolase (Gao and Simon, 2005) appeared to contain an RE hydrolase inhibitor. These pools were further subdivided so that additional rounds of screening were carried out using pools of decreasing complexity. Those used in screening rounds 2, 3, 4, and 5 contained 1,000, 100, 16 clones and one clone, respectively (see Materials and Methods). In round 3, transfectants of sub-pools were assayed by Western blot for GS2 lipase expression. Pools showing reduced RE hydrolase activity and GS2 expression equivalent to pGS2 control transfectants were further subdivided (data not shown). Any pools that had reduced the expression of GS2 lipase were not further evaluated. In this way, a single cDNA encoding a GS2 lipase inhibitor was identified.

Figure 2 shows the results of a representative set of RE hydrolase assays carried out with 293T cells co-transfected either with 10 ng pGS2 and 2 μg empty vector or with 10 ng pGS2 and 2 μg of the RE hydrolase inhibitor clone. The homogenates prepared from transfectants containing pGS2 and empty vector hydrolyzed 35% of the added RE

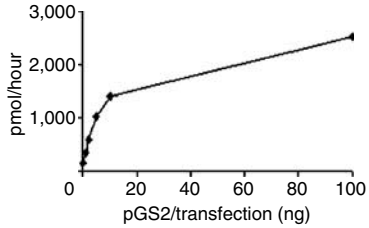

Figure 1. pGS2 expression in transfected 293T cells. Shown is the RE hydrolase activity of homogenates made from transfections of 293T cells with increasing concentrations (0–100 ng) of pGS2 is shown. RE hydrolysis was measured by incubating 50 μl of each homogenate with 33 μM retinyl palmitate (RE) in a total volume of 200 μl. Reactions were terminated after 1 hour at 37°C. Retinoids were extracted and resolved by HPLC. RE and product retinol were detected by retention time and absorbance at 326 nm.

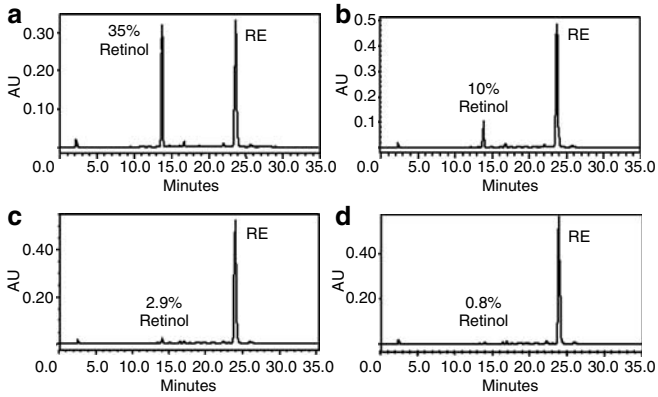

Figure 2. Reduced REH activity in co-transfections with GS2 lipase and TIP47. The RE hydrolysis catalyzed by 1 hour 37°C incubations of 33 μM retinyl palmitate (RE) with 100 μl of homogenates of 293T cells transfected with (**a**) 10 ng GS2 lipase (**b**) 10 ng GS2 lipase and 2 μg TIP47, (**c**) 2 μg empty vector or (**d**) 2 μg TIP47 is shown. The percentage of RE hydrolyzed to retinol is indicated. Retinoids were extracted and resolved by HPLC with detection at 326 nm; 1% = 66 pmol.

(2,310 pmol/hour), whereas the homogenates prepared from transfectants containing pGS2 and the inhibitor hydrolyzed 10% of the added RE (660 pmol/hour). The average inhibition of RE hydrolysis by TIP47 in co-transfections was 67% (±6%), $n=8$. A similar three-fold decrease in RE hydrolase activity was observed in homogenates prepared from transfectants containing the inhibitor alone when compared to homogenates from transfectants containing empty vector. Similar to the reduction in activity of ectopically expressed GS2 lipase, TIP47 inhibited the endogenous RE hydrolase activity of 293T cells by 70% (±7%), $n=7$.

To determine whether the observed inhibition was owing to TIP47 suppression of GS2 expression, altered subcellular localization, misfolding, or other indirect mechanisms, we carried out an additional series of experiments. In these experiments, homogenates prepared from pGS2 transfectants were mixed either with homogenates prepared from empty-vector transfectants or from pTip47 transfectants. A representative experiment is shown in Figure 3. The results obtained with mixtures of transfectants were similar to those obtained using co-transfectants. Mixtures of homogenates prepared from pGS2 transfectants with homogenates from empty vector or from pTip47 transfectants hydrolyzed 14% of the added RE (920 pmol/hour) or 3.6% of the added RE (238 pmol/hour), respectively. The average inhibition of RE hydrolysis by pTIP47 in these experiments was 67% (±10%), $n=17$.

We then determined the sequence of the inhibitor cDNA and found to be identical to TIP47 (Accession number: BC019278). TIP47 is a 434-residue protein that serves as a cargo protein involved in the movement of the cation-dependent-MPR and cation-independent-MPR between the late endosomes and the *trans*-Golgi network (Diaz and Pfeffer, 1998; Orsel *et al.*, 2000). More recently, TIP47 has also been found with lipid bodies and with the plasma membrane (Wolins *et al.*, 2001, 2005; Miura *et al.*, 2002; Brasaemle *et al.*, 2004; Robenek *et al.*, 2005a–c).

Dose-dependent inhibition GS2 lipase activity by TIP47

The inhibition of RE hydrolase activity was next measured as a function of increasing concentrations of TIP47. As shown in Figure 4, at 2 μl TIP47 homogenate/reaction RE hydrolysis was inhibited by 34%. Using three times that amount of TIP47 increased inhibition to only 44%, whereas 30 μl inhibited the reaction by about 60%. Thus, under the conditions of the assay, homogenates containing TIP47 were unable to completely abolish RE hydrolase activity.

TIP47 inhibits multiple RE hydrolase activities

To determine whether TIP47 inhibitory activity was unique to GS2 lipase, we evaluated its impact on the RE hydrolase activities of homogenates prepared from pHSL transfectants and from SCC13. Before the addition of substrate RE, each of these homogenates was combined either with homogenates prepared from empty vector transfectants or from pTIP47

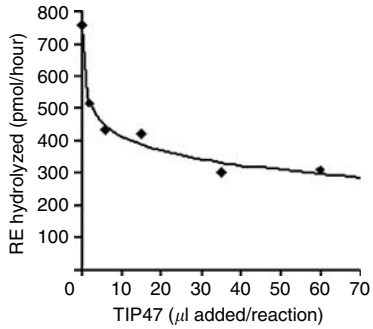

Figure 4. Dose dependence of RE hydrolase inhibition. RE hydrolysis was measured in 1 hour, 37°C reactions of homogenates of pGS2 transfectants (10 μl) mixed with increasing amount of homogenates of pTIP47 transfectants (0–60 μl). The volume of homogenate was held at 70 μl by adjusting samples with homogenates of empty vector transfectants. The data represent the mean of duplicates; the difference between duplicates was less than 13%.

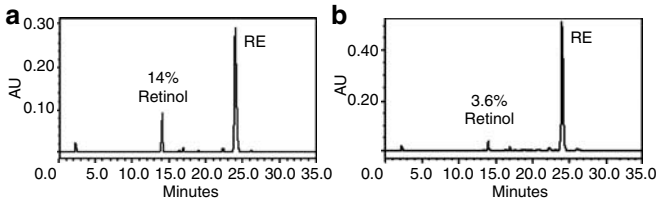

Figure 3. TIP47-containing homogenates inhibit GS2 lipase REH activity. Homogenates were prepared from 293T cells transfected with 2 μg of either pGS2, pTIP47 vector, or empty vector. RE hydrolysis was measured using 10 μl of the GS2 lipase homogenate mixed either with (**a**) 90 μl of empty vector or (**b**) 90 μl of TIP47. Both reactions were carried out for 1 hour at 37°C incubations using 33 μM retinylpalmitate (RE) as substrate. The percentage of RE hydrolyzed to retinol is indicated. Retinoids were extracted and resolved by HPLC with detection at 326 nm. 1% = 66 pmol.

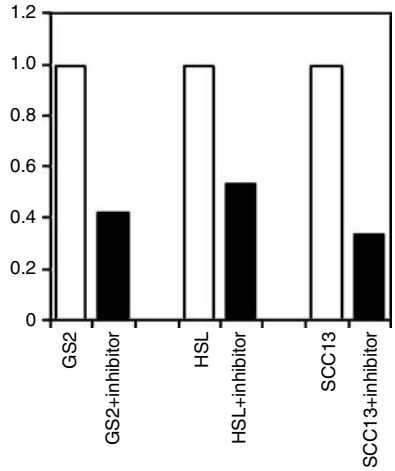

Figure 5. TIP47 inhibits multiple hydrolytic activities. RE hydrolysis was measured in homogenates prepared from pGS2 transfectants, pHSL transfectants, and from homogenates of SCC13 mixed either with homogenates of empty vector transfectants or pTIP47 transfectants. Reactions were carried out for 1 hour at 37°C. For comparison, the hydrolysis detected in mixtures with empty vector transfectants was set at one. For GS2 lipase, hHSL, and SCC13, the rates with empty vector were 521, 3,300, and 469 pmol/hour. The data represent the mean of duplicates; the difference between duplicates was less than 2%.

transfectants. As shown in Figure 5, TIP47 homogenates inhibited the RE hydrolase activity of GS2 lipase, HSL, and SCC13 by 50–60%. Thus, TIP47 has the capability of inhibiting multiple RE hydrolase activities.

Regions of TIP47 required for inhibition of RE hydrolysis

To determine the region(s) of TIP47 essential for inhibitory function, we constructed a series of TIP47 deletion and truncation mutants and compared their inhibitory activity to that of full-length TIP47. The results are shown in Figure 6, which also contains a map of the major α-helices found by X-ray crystallography that form a hydrophobic surface on TIP47 (Hickenbottom *et al.*, 2004). The 11-mer repeat that is shown is an amphipathic helix that is homologous to the lipid-binding domains of apolipoprotein A and synuclein (Bussell and Eliezer, 2003), and harbors residues essential for binding Rab9 (Hanna *et al.*, 2002).

We evaluated the amino-terminal half of TIP47 first. In this series of experiments, full-length TIP47 inhibited the RE hydrolysis by 67% (±10%, $n=10$), whereas the N-terminal truncation mutants comprising residues 1–258*, 1–218*, 17–258*, 78–288*, and 107–258* inhibited hydrolysis by 80, 75, 76, 67, and 50%, respectively. Residues 139–258* had no inhibitory activity, suggesting that the amino-terminus harbored sequences necessary and sufficient for RE hydrolase inhibition. However, the carboxyl portion of TIP47 was also effective. Similar to full-length TIP47, peptides comprising residues 139–434, 139–413, 139–380, 139–352, 219–434, and 239–434 inhibited GS2 lipase activity by 50–67%. Deletions of part, or all, of the α3 and α4 helical surface resulted in the progressive loss of inhibitory function. Residues 260–434 and 279–434 inhibited RE hydrolysis by only 16–22%, and residues 329–434 actually increased GS2 lipase activity by 28–30% in each of two separate experiments.

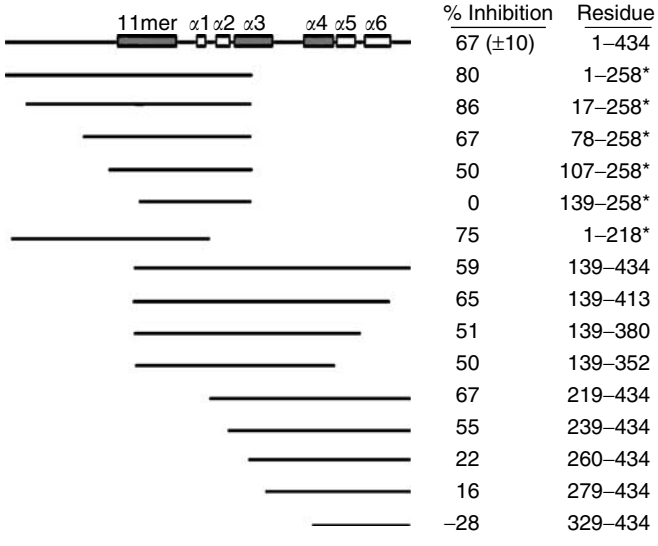

Figure 6. Truncation and deletion analyses of TIP47. Wild-type TIP47 with proposed structural elements and the generated deletion mutants assayed for GS2 lipase-driven RE hydrolysis is shown. GS2 lipase activity was measured with homogenates prepared from pGS2 transfectants mixed with homogenates from empty vector transfectants (100% activity equivalent to 0% inhibition) or with homogenates from transfectants of the indicated TIP47 deletion mutants.

Internal deletion mutations identify a region of TIP47 essential for activity

To determine whether either region identified as essential for RE hydrolase inhibition in the truncation analyses was functional in the context of the full-length molecule, we constructed and tested internal deletion mutants. As shown in Figure 7a, deletions of the 11-mer region (1–434Δ118–180) alone only minimally affected TIP47 function. Inhibition was reduced from 67% (±10%), $n=17$ to 47% (±15%), $n=7$. In contrast, deletion of the α3 and α4 helices (1–434Δ239–351) significantly reduced TIP47 effectiveness. This internal deletion mutant inhibited RE hydrolase activity by only 19% (±25%), $n=7$. The double deletion (1–434Δ118–180Δ239–351) abolished almost all TIP47 function; this deletion reduced activity by 6% (±3%), $n=7$. As shown in Figure 7b, the expression of the mutants was comparable to that of the full-length protein (Figure 7b). Therefore, differences in the inhibitory activities reflect differences in function rather than expression.

DISCUSSION

We have found that homogenates prepared from pTip47 transfectants inhibit RE hydrolysis catalyzed by GS2 lipase, hHSL, and homogenates of SCC13. TIP47 is a member of the PAT (for perilipin, adipocyte differentiation-related protein, and TIP47) family, which includes perilipin, adipophilin, as well as a number of other related proteins showing various degrees of homology (e.g. S3–12, and the *Drosophila* protein LSDP-2) (Miura *et al.*, 2002). Each of these proteins can associate with lipid droplets, especially upon lipid loading of cells (Londos *et al.*, 2005). As the PAT proteins contain 11-mer repeat regions within their amino-termini, which are similar to those found in the lipid-binding regions of apolipoprotein A and synuclein (Bussell and Eliezer, 2003), it had been postulated that these repeats serve a similar

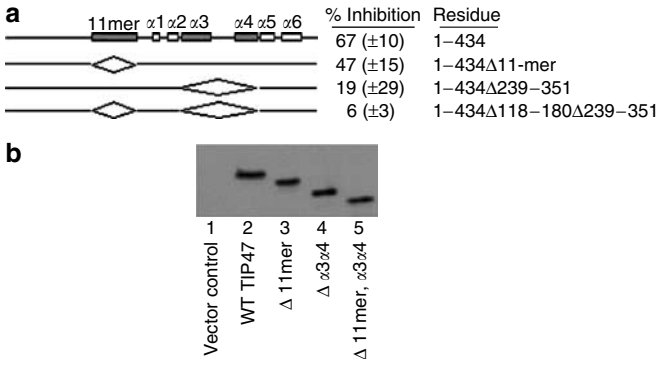

Figure 7. Internal deletions of TIP47. (**a**) TIP47 deleted of the 11-mer(Δ118–180), of the region containing helices α_3–α_4(Δ239–351), or of both regions were constructed and tested for their ability to suppress GS2-driven RE hydrolysis as in Figure 5. (**b**) The expression of TIP47 and each mutant was evaluated by Western blot.

function in the PAT proteins. However, deletions of the 11-mer repeats do not eliminate lipid binding of the PAT proteins and recent reports suggest the participation of other regions capable of lipid binding (Garcia *et al.*, 2003; McManaman *et al.*, 2003; Nakamura and Fujimoto, 2003; Subramanian *et al.*, 2004).

The most studied of the PAT proteins is perilipin. It is expressed as multiple splice variants with identical carboxyl-termini (406 amino acids) (Lu *et al.*, 2001). Perilipin A is found in adipocytes and steroidogenic cells. Perilipin B is found primarily in adipocytes and perilipin C is found primarily in steroidogenic cells. The most studied perilipin, perilipin A, controls HSL activity at the surface of lipid droplets. This function is modulated by protein kinase A-mediated phosphorylation of both perilipin and HSL. In its unphosphorylated form, it shields the lipid droplet from HSL activity. In its phosphorylated form, perilipin enhances the interaction with phosphorylated HSL (and possibly other lipases) and stimulates lipolysis (Londos *et al.*, 2005).

The second member of the family, adipophilin, is the human analog of mouse adipocyte differentiation-related protein. It is expressed in multiple lipid-containing cells, including adipocytes, mammary epithelia, hepatocytes, and muscle cells (Jiang and Serrero, 1992; Heid *et al.*, 1998) where it localizes to lipid droplets (Brasaemle *et al.*, 1997). The function(s) of adipophilin are now being studied and may be related to lipid droplet biogenesis or activity in a cell type-dependent manner (Larigauderie *et al.*, 2004; Londos *et al.*, 2005). For example, in late adipocyte differentiation as droplet size increases, adipophilin is replaced by perilipin (Londos *et al.*, 1999; Imamura *et al.*, 2002), whereas in mammary epithelia, it remains associated with the fat globule and is secreted (Heid *et al.*, 1996).

TIP47 differs in a number of ways from all the other PAT proteins. First, as stated above, one of its unique functions is as a cargo protein for cation-independent-MPR/IGFR-II and cation-dependent-MPR (Schweizer *et al.*, 1997; Diaz and Pfeffer, 1998; Orsel *et al.*, 2000). Although the carboxyl-terminus is capable of binding of the MPRs, cargo function requires the amino–terminus, suggesting a role for oligimerization. In addition, the amino-terminus contains residues (161–169) essential for binding of Rab9 to TIP47 (Hanna *et al.*, 2002). Rab9 increases the affinity of cation-independent-MPR to TIP47 three-fold (Carroll *et al.*, 2001; Barbero *et al.*, 2002). The interaction with cation-independent-MPR/IGFR-II places TIP47 as a regulator of signals originating from the MPR/IGF-II receptor. This receptor also binds urokinase-type plasminogen activator receptor, plasminogen, and retinoic acid, and impacts transforming growth factor-β activation, apoptosis, and tumor suppression (Ghosh *et al.*, 2003; Jessen *et al.*, 2005). Second, in contrast to perilipin and adipophilin, a significant fraction of TIP47 is found in the cytoplasm, as well as on the surface of and within lipid droplets (Wolins *et al.*, 2001, 2005; Miura *et al.*, 2002; Ghosh *et al.*, 2003; Brasaemle *et al.*, 2004; Robenek *et al.*, 2005a–c). Third, in contrast to the other PAT proteins in the mouse, TIP47 expression is not increased in response to PPARγ agonists; rather, it appears to be expressed constitutively in multiple tissues (Dalen *et al.*, 2004). What governs TIP47 distribution between multiple cellular compartments and therefore helps govern its multiple functions is not yet known. Given its ability to interact with proteins and lipids, regulation by both or either molecular type is possible.

The TIP47 residues required for oligimerization, cargo function, and Rab9 binding (residues 161–169) (Carroll *et al.*, 2001; Hanna *et al.*, 2002; Sincock *et al.*, 2003) are not essential for RE hydrolase inhibition in the context of full-length TIP47. Rather, as shown by truncation mutant and internal deletion mutant analyses (Figures 6 and 7), the essential region is encompassed by the carboxyl-terminal $\alpha_{3/4}$-helices; these helices supports $>60\%$ of the inhibitory activity. However, truncation mutants comprising only the amino-terminal half of TIP47 also inhibited RE hydrolase activity. Therefore, we cannot exclude participation of this region in RE hydrolase inhibition under certain, as yet undefined, circumstances.

Our findings contribute to the growing list of functions carried out by members of the PAT family. Both normal epidermal keratinocytes and the transformed cell line SCC13, the line used to generate our cDNA library (Gao and Simon, 2005), express TIP47 mRNA to similar levels as determined by reverse transcriptase-polymerase chain reaction using primers TIP47*Kpn*1(721–737) and TIP47*Xho*1(1,473–1,457) (data not shown). We therefore speculate that TIP47 may participate in epidermal keratinocyte regulation of RE storage pools and provide an additional mechanism to adjust the levels of substrate retinol for the production of its downstream metabolite atRA. In the epidermis and in cultured epidermal keratinocytes, atRA concentration determines whether the expression of markers of terminal differentiation such as profilaggrin will be promoted or inhibited (Asselineau *et al.*, 1989). Its maintenance within a narrow concentration range is critical for normal tissue morphogenesis and determines whether the tissue will be parakeratotic or orthokeratotic. In part, this is owing to the impact of atRA on keratinocyte proliferation, choice of differentiation program, as well as on the expression of differentiation markers (Asselineau *et al.*, 1989; Tur *et al.*, 1995; Eichner *et al.*, 1996; Gibbs *et al.*, 1996). It will be interesting to determine whether the role of TIP47 in the epidermis is governed by differentiation state. In the basal layer containing proliferative cells, TIP47 may function as a modulator of cation-independent-MPR/IGFR-II-mediated regulation of cell growth or migration, via its role as a cargo protein. In the suprabasal layers, TIP47 may function as a modulator of differentiation, via its role as an inhibitor of RE hydrolases and lipases. The mechanism(s) and extent to which TIP47 and GS2 lipase coordinately regulate these processes is the subject of current investigation.

MATERIALS AND METHODS

Approvals

The committees on research compliance for use of recombinant DNA, and radioactive materials, the ethic committee of the State University of New York at Stony Brook approved all described studies.

Cell culture 293T cells, a human embryonic kidney cell line containing the large T antigen of SV40, were grown in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Salt Lake City, UT) without antibiotics. SCC13, a transformed epidermal keratinocyte line isolated from a head and neck tumor (Rheinwald and Beckett, 1981), was grown with lethally irradiated 3T3 cells, harvested and passaged as described previously (Rheinwald and Green, 1975; Randolph and Simon, 1993). For experiments, cultures were harvested at 2 days post-confluence.

Transient transfection of 293T cells

Transfections were carried out according to the methods described by Gao and Simon (2005). For library screening and analyses of single clones, 293T cells were co-transfected with 2 μg of the indicated pool or clone in combination with 1 or 10 ng of the GS2 vector (pGS2), respectively. To generate pTIP47 transfectants, 293T cells were transfected with 2 μg of the pTIP47 expression vector (see Materials and Methods section entitled ''Construction of TIP47 deletion mutants'') and to generate pHSL transfectants, 293T cells were transfected with 2 μg of the pHSL expression vector (Gao and Simon, 2005).

Preparation of cell homogenates

Cultures were washed with calcium-free phosphate-buffered saline (PBS), pH 7.4, scraped from the plastic surface, and collected by centrifugation at 3,000 r.p.m. After one additional PBS wash, cells were resuspended in 250 μl PBS and sonically disrupted using 15 bursts of a Branson Sonifer. Protein concentrations were determined by using Bio-Rad Protein Assay (Bio-Rad Life Sciences, Hercules, CA) with bovine serum albumin as a standard.

RE hydrolase assays

RE hydrolysis was measured in homogenates of 293T cells co-transfected with pGS2 and the indicated GS2 lipase inhibitor, and in homogenates prepared by mixing homogenates from 293T cells transfected with GS2 lipase inhibitor, with homogenates from pGS2 transfectants or p-hHSL transfectants. For assays of co-transfectants, 100 μl of cell homogenates were used. Unless otherwise indicated, assays of homogenate mixtures contained 90 μl of the GS2 lipase inhibitor and either 10 μl of pGS2 or 1 μl of p-hHSL.

The final volumes were adjusted to 200 μl with PBS. The pGS2 and p-hHSL constructs have been described (Gao and Simon, 2005). Control homogenates were prepared from 293T cells transfected with empty vector.

Reaction mixtures were incubated at 37°C for 1 hour with gentle shaking. Cell extracts, boiled for 1 minute, were used to measure non-enzymatic conversions. Retinoids were extracted and resolved by reverse-phase high-pressure liquid chromatography with photodiode array detection as described previously (Randolph and Simon, 1993) using a Waters 2996 photodiode array detector equipped with automated injection. Retinoids were identified by retention time and absorbance at 326 nm. Spectral analyses were carried out for verification.

Screening of cDNA expression libraries for GS2 lipase inhibitor

For the identification and isolation of the GS2 lipase inhibitor, we used the two cDNA expression libraries described previously (Gao and Simon, 2005). The libraries comprise several hundred pools of cDNA-containing plasmids, with each pool containing 4,000 cDNA clones. In a previous experiment in which 120 pools were assessed for RE hydrolase activity, 48 were found to have activities below empty vector control samples. To increase the sensitivity of detection, we chose to evaluate these 48 pools by co-transfection with pGS2 (see above). Two pools (nos. 2 and 53) showed inhibitor activity.

In the next round screening, 10,000 clones from each inhibitor-containing pool (nos. 2 and 53) were evaluated. From each pool, 100 clones were plated onto each of 100 Luria Broth plates containing 100 μg/ml ampicillin. The bacteria from each plate were collected separately and placed into 5 ml of Luria Broth. Four milliliters were saved for later use, and 1 ml from each of 10 plates was pooled to generate a total of 20 combined sub-pools each with 1,000 clones. Plasmid DNA was prepared and co-transfected with pGS2 for each of the combined sub-pools. Two of the combined sub-pools showed inhibitor activity: no. 1, derived from pool no. 2; and no. 20, derived from pool; and no. 53.

For the third round of screening, plasmid DNA was prepared from each of the sub-pools that was used to produce combined sub-pool nos. 1 and 20. Each of these 20 plasmid DNA preparations (100 clones/preparation) was used to assay RE hydrohysis inhibition. At this stage, the expression of GS2 lipase was also checked by Western blot analysis (data not shown; Gao and Simon, 2005). Three sub-pools showed inhibitor activity and expressed GS2 lipase at levels similar to control homogenates. The sub-pool yielding the greatest REH inhibition (80%) was used for the next round of screening, and this sub-pool originated from pool no. 53.

For the fourth round of screening, 384 single clones from the inhibitory sub-pool were individually cultured in 2.5 ml Luria Broth with 100 μg/ml ampicillin. One hundred and fifty microliters from each of the 16 cultures were mixed, DNA was prepared from each of the 24 mixtures, and RE hydrolysis inhibition was evaluated. For final screening, each of the 16 clones of the cDNA mixture causing REH inhibition was evaluated.

Construction of TIP47 deletions

We identified a single cDNA from a random cDNA library that inhibited RE hydrolysis by GS2 lipase. This cDNA, encoding TIP47, was cloned into the *Eco*RI site of pCMV-Sport6 (Invitrogen, Carlsbad, CA). To construct pTip47(1–258), we deleted the 1.4 kb *Hin*dIII fragment from pTip47. When transfected into mammalian cells, this plasmid (pTip47(1–258)) is expressed as a fusion protein comprising residues 1–258 of TIP47 linked to 12 amino acids encoded by the vector. The sequence of the vector derived 12 amino acids is TRTQLSCTKWSL. The vector is renamed pTip47(1–258*) to indicate the additional expressed amino-acid residues.

To construct external deletions, plasmid DNA of pTip47 was used as a template in PCR reactions with primer sets shown in Table 1 and high-fidelity *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). To construct pTip47(17–258*), pTip47(78–258*), pTip47(107–258*), pTip47(139–258*), and pTip47(1–218*), the corresponding PCR products were purified, digested with restriction enzymes *Kpn*I and *Hin*dIII and cloned into pCMV-Sport6 at the *Kpn*I and *Hin*dIII sites. To construct pTip(219–434), pTip(239–434), pTip(260–434), pTip(279–434), pTip(329–434), pTip(139–434), pTip(139–413), pTip(139–380), and pTip(139–352), the PCR products

Table 1. Primers used to construct truncation and deletion mutants of Tip47

Construct	Primer name	Primer sequence
pTip47(17–258)12aa	Tip47*Kpn*I(115–130)	5′-ggggtaccatggaagaaccggtacagc-3′
	Tip47(877–863)	5′-cagcagagcctcctg-3′
pTip47(78–258)12aa	Tip47*Kpn*I(298–313)	5′-ggggtaccatggctcagccgatcctctc-3′
	Tip47(877–863)	5′-cagcagagcctcctg-3′
pTip47(107–258)12aa	Tip47*Kpn*I(386–392)	5′-ggggtaccatgctccccatcctgcagc-3′
	Tip47(877–863)	5′-cagcagagcctcctg-3′
pTip47(139–258)12aa	Tip47 *Kpn*I(481–497)	5′-ggggtaccatggccaaggacacggtggc-3′
	Tip47(877–863)	5′-cagcagagcctcctg-3′
	Sp6 promoter primer	5′-atttaggtgacactatag-3′
pTip47(1–218)12aa	Tip47*Hin*dIII(720–705)	5′-ccttaaagcttcagggatgtggcgatg-3′
	Tip47*Kpn*I721)	5′-ggggtaccatggatggcttcgacgtcgc-3′
pTip47(219–434)	Tip47*Xho*I(1,473)	5′-gccctcgaggagtggctagaaaa-3′
	Tip47*Kpn*I(781)	5′-ggggtaccatgctgggctccctgtcggag-3′
pTip47(239–434)	Tip47*Xho*I(1,473)	5′-gccctcgaggagtggctagaaaa-3′
	Tip47*Kpn*I(844)	5′-ggggtaccatggccaccaagcagagggc-3′
pTip47(260–434)	Tip47*Xho*I(1,473)	5′-gccctcgaggagtggctagaaaa-3′
	Tip47*Kpn*I(901)	5′-ggggtaccatggaaactgtcaag-3′
pTip47(279–434)	Tip47*Xho*I(1,473)	5′-gccctcgaggagtggctagaaaa-3′
	Tip47*Kpn*I(1,052)	5′-ggggtaccatgttccgggacattg-3′
pTip47(329–434)	Tip47*Xho*I(1,473)	5′-gccctcgaggagtggctagaaaa-3′
	Tip47*Kpn*I(481–497)	5′-ggggtaccatggccaaggacacggtggc-3′
pTip47(139–434)	Tip47*Xho*I(1,473–1,457)	5′-gccctcgaggagtggctagaaaa-3′
	Tip47*Kpn*I(481–497)	5′-ggggtaccatggccaaggacacggtggc-3′
pTip47(139–413)	Tip47*Xho*I(1,305–1,287)	5′-gccctcgagttaaggtgtgttctgggccac-3′
	Tip47*Kpn*I(481–497)	5′-ggggtaccatggccaaggacacggtggc-3′
pTip47(139–380)	Tip47*Xho*I(1,206–1,181)	5′-gccctcgagttactggaaggagtggatgct-3′
	Tip47*Kpn*I(481–497)	5′-ggggtaccatggccaaggacacggtggc-3′
pTip47(139–352)	Tip47*Xho*I(1,122–1,094)	5′-gccctcgagttaggggaggccctgaatgct-3′
	Sp6 promoter primer	5′-atttaggtgacactatag-3′
pTip47(1–258)12aa	Tip47Δ11mers(−)	5′-caccatctggcccaagcggaccttctccgtgggctg-3′
	Tip47Δ11mers(+)	5′-cagcccacggagaaggtccgcttgggccagatggtg-3′
	Δ11–mers Tip47*Xho*I(1,473–1,457)	5′-gccctcgaggagtggctagaaaa-3′
pTip47(184–434)	Tip47*Kpn*I(616–633)	5′-ggggtaccatggtgctgagtggggtc-3′
	Tip47Δα3–α4(−)	5′-gtccttcacattggtgggacgtacgaagtagctctg-3′
	Δα3–α4 Tip47Δα3–α4(+)	5′-cagagctacttcgtacgtcccaccaatgtgaaggac-3′
	Tip47*Xho*I(1,473–1,457)	5′-gccctcgaggagtggctagaaaa-3′

were purified, digested with restriction enzymes *Kpn*I and *Xho*I, and cloned into pCMV-Sport6 at the *Kpn*I and *Xho*I site.

Two rounds of PCR were carried out to construct the internal deletion in pTip47 (1258*)Δ11-mers. In the first-round PCR, pTip47 was used as template. Two PCR reactions were carried out, one with Sp6 promoter/Tip47Δ11mers(−) primers, and the other with Tip47Δ11mers(+)/Tip47 (877–863) primers. The PCR products were purified from agarose gels, mixed, and used as template for the next round of PCR, in which, Sp6 promoter/Tip47 (877–863) were used. Primer Tip47Δ11-mers (+) and Tip47Δ11-mers (–) are complementary primers. The first-half sequence of Tip47Δ11-mers (+) primer is the Tip47 cDNA sequence from 400 to 417, and the second half is Tip47 cDNA sequence from 604 to 621. The second- round PCR product was purified, digested with *Kpn*I and *Hin*dIII, and cloned into pCMV-Sport6 at the *Kpn*I and *Hin*dIII sites. The product is pTip47 (1–258*)Δ11-mers, in which the Tip47 amino-acid sequence from 118 to 179 is deleted. Using a similar approach, pTip47 (184–434)Δα3–α4 was constructed with the primers shown in Table 1. The secondround PCR products were cloned into *Knp*I and *Xho*I sites of pCMV-Sport6. Tip47 amino-acid sequence from 244 to 348 is deleted when pTip47(184–434)Δα3–α4 is expressed in mammalian cells.

The deletion in pTip47Δ11-mers was constructed by replacing the 0.6 kb *Kpn*I/*Acc*I fragment of pTip47 with the 0.4 kb *Kpn*I/*Acc*I fragment from pTip47(1–258*) Δ11-mers. The deletion in pTip47-Δα3–α4 was constructed by replacing the 1.6 kb *Acc*I/*Xho*I fragment of pTip47 with the 0.84 kb *Acc*I/*Xho*I fragment from pTip47(184–434)Δα3–α4, and the double deletion in pTip47Δ11—mersΔα3–α4 was constructed by cloning the 0.4 kb *Kpn*I/*Acc*I fragment of pTip47(1258*)Δ11-mers into pTip47(184–434)Δα3–α4 at the *Kpn*I and *Acc*I sites.

Western blot analyses

Proteins were extracted and resolved on a 4–20% acrylamide gel and transferred to nitrocellulose. The nitrocellulose was incubated for 1 hour with 5% powdered milk in PBS containing 0.2% Tween (PBS–Tween), and then incubated with either rabbit anti-human GS2 lipase (prepared using full-length GS2 lipase by Sigma-Genosys, Woodlands, TX) diluted 1:200 or with anti-TIP47 antibody (C-20; Santa Cruz, Santa Cruz, CA) diluted 1:600 overnight at 4°C. Immunoblots were washed twice with PBS, three times with PBS–Tween, and then incubated with secondary goat anti-immunoglobulin G-horse radish peroxidase for 1 hour at room temperature. Antigens were visualized using Pierce Supersignal WestPico Chemiluminescent substrate according to the manufacturer's instructions (Pierce, Rockford, IL).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by a grant from Unilever Research, USA to M.S. M.S. is a consultant to Unilever.

REFERENCES

Asselineau D, Bernard BA, Bailly C, Darmon M (1989) Retinoic acid improves epidermal morphogenesis. *Dev Biol* 133:322–35

Barbero P, Bittova L, Pfeffer SR (2002) Visualization of Rab9-mediated vesicle transport from endosomes to the *trans*-Golgi in living cells. *J Cell Biol* 156:511–8

Bastien J, Rochette-Egly C (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 328:1–16

Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, Londos C (1997) Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res* 38:2249–63

Brasaemle DL, Dolios G, Shapiro L, Wang R (2004) Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J Biol Chem* 279:46835–42

Bussell R Jr, Eliezer D (2003) A structural and functional role for 11-mer repeats in alphasynuclein and other exchangeable lipid binding proteins. *J Mol Biol* 329:763–78

Carroll KS, Hanna J, Simon I, Krise J, Barbero P, Pfeffer SR (2001) Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. *Science* 292:1373–6

Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H *et al.* (2004) Adipose tissue expression of the lipid droplet-associating proteins S3–12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. *Diabetes* 53:1243–52

Diaz E, Pfeffer SR (1998) TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. *Cell* 93:433–43

Eichner R, Gendimenico GJ, Kahn M, Mallon JP, Capetola RJ, Mezick JA (1996) Effects of long-term retinoic acid treatment on epidermal differentiation *in vivo*: specific modification in the programme of terminal differentiation. *Br J Dermatol* 135:687–95

Gao J, Simon M (2005) Identification of a novel keratinocyte retinyl ester hydrolase as a transacylase and lipase. *J Invest Dermatol* 124:1259–66

Garcia A, Sekowski A, Subramanian V, Brasaemle DL (2003) The central domain is required to target and anchor perilipin A to lipid droplets. *J Biol Chem* 278:625–35

Ghosh P, Dahms NM, Kornfeld S (2003) Mannose 6-phosphate receptors: new twists in the tale. *Nat Rev Mol Cell Biol* 4:202–12

Gibbs S, Backendorf C, Ponec M (1996) Regulation of keratinocyte proliferation and differentiation by all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 1,25-dihydroxy vitamin D3. *Arch Dermatol Res* 288:729–39

Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E *et al.* (2002) Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. *J Biol Chem* 277:4806–15

Hanna J, Carroll K, Pfeffer SR (2002) Identification of residues in TIP47 essential for Rab9 binding. *Proc Natl Acad Sci USA* 99:7450–4

Harrison EH (1998) Lipases and carboxylesterases: possible roles in the hepatic metabolism of retinol. *Annu Rev Nutr* 18:259–76

Heid HW, Moll R, Schwetlick I, Rackwitz HR, Keenan TW (1998) Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell Tissue Res* 294:309–21

Heid HW, Schnolzer M, Keenan TW (1996) Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. *Biochem J* 320:1025–30

Hickenbottom SJ, Kimmel AR, Londos C, Hurley JH (2004) Structure of a lipid droplet protein; the PAT family member TIP47. *Structure (Cambridge)* 12:1199–207

Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N *et al.* (2002) ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *Am J Physiol Endocrinol Metab* 283:E775–783

Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B, Gross RW (2004) Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem* 279:48968–75

Jessen KA, English NM, Yu Wang J, Maliartchouk S, Archer SP, Qiu L *et al.* (2005) The discovery and mechanism of action of novel tumor-selective and apoptosis-inducing 3,5-diaryl-1,2,4-oxadiazole series using a chemical genetics approach. *Mol Cancer Ther* 4:761–71

Jiang HP, Serrero G (1992) Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. *Proc Natl Acad Sci USA* 89:7856–60

Jurukovski V, Simon M (1999) Reduced lecithin:retinol acyl transferase activity in cultured squamous cell carcinoma lines results in increased substrate-driven retinoic acid synthesis. *Biochim Biophys Acta* 1436:479–90

Kurlandsky SB, Duell EA, Kang S, Voorhees JJ, Fisher GJ (1996) Auto-regulation of retinoic acid biosynthesis through regulation of retinol esterification in human keratinocytes. *J Biol Chem* 271: 15346–15352

Lake AC, Sun Y, Li JL, Kim JE, Johnson JW, Li D *et al.* (2005) Expression, regulation, and triglyceride hydrolase activity of Adiponutrin family members. *J Lipid Res* 46:2477–87

Larigauderie G, Furman C, Jaye M, Lasselin C, Copin C, Fruchart JC *et al.* (2004) Adipophilin enhances lipid accumulation and prevents lipid efflux from THP-1 macrophages: potential role in atherogenesis. *Arterioscler Thromb Vasc Biol* 24:504–10

Lee W-C, Salido E, Yen PH (1994) Isolation of a new gene GS2 (DXS1283E) from a CpG island between STS and KAL1 on Xp22.3. *Genomics* 22:372–6

Londos C, Brasaemle DL, Schultz CJ, Segrest JP, Kimmel AR (1999) Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin Cell Dev Biol* 10:51–8

Londos C, Sztalryd C, Tansey JT, Kimmel AR (2005) Role of PAT proteins in lipid metabolism. *Biochimie* 87:45–9

Lu X, Gruia-Gray J, Copeland NG, Gilbert DJ, Jenkins NA, Londos C *et al.* (2001) The murine perilipin gene. The lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mamm Genome* 12:741–9

Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. *Cell* 83:841–50

McManaman JL, Zabaronick W, Schaack J, Orlicky DJ (2003) Lipid droplet targeting domains of adipophilin. *J Lipid Res* 44:668–73

Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C *et al.* (2002) Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, *Drosophila*, and *Dictyostelium*. *J Biol Chem* 277:32253–7

Nakamura N, Fujimoto T (2003) Adipose differentiation-related protein has two independent domains for targeting to lipid droplets. *Biochem Biophys Res Commun* 306:333–8

Napoli JL (1986) Retinol metabolism in LLC-PK1 cells. Characterization of retinoic acid synthesis by an established mammalian cell line. *J Biol Chem* 261:13592–7

Napoli JL, Boerman MH, Chai X, Zhai Y, Fiorella PD (1995) Enzymes and binding proteins affecting retinoic acid concentrations. *J Steroid Biochem Mol Biol* 53:497–502

Orsel JG, Sincock PM, Krise JP, Pfeffer SR (2000) Recognition of the 300-kDa mannose 6-phosphate receptor cytoplasmic domain by 47-kDa tail-interacting protein. *Proc Natl Acad Sci USA* 97:9047–51

Randolph RK, Simon M (1993) Characterization of retinol metabolism in cultured human epidermal keratinocytes. *J Biol Chem* 268:9198–205

Randolph RK, Simon M (1998) Dermal fibroblasts actively metabolize retinoic acid but not retinol. *J Invest Dermatol* 111:478–84

Randolph RK, Winkler KE, Ross AC (1991) Fatty acyl CoA-dependent and -independent retinol esterification by rat liver and lactating mammary gland microsomes. *Arch Biochem Biophys* 288:500–8

Rheinwald JG, Beckett MA (1981) Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas. *Cancer Res* 41:1657–63

Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–43

Robenek H, Lorkowski S, Schnoor M, Troyer D (2005a) Spatial integration of TIP47 and adipophilin in macrophage lipid bodies. *J Biol Chem* 280:5789–94

Robenek H, Robenek MJ, Buers I, Lorkowski S, Hofnagel O, Troyer D *et al.* (2005b) Lipid droplets gain PAT family proteins by interaction with specialized plasma membrane domains. *J Biol Chem* 280:26330–8

Robenek H, Robenek MJ, Troyer D (2005c) PAT family proteins pervade lipid droplet cores. *J Lipid Res* 46:1331–8

Ross AC (2003) Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic acid oxidation. *J Nutr* 133:291S–6S

Rydel TJ, Williams JM, Krieger E, Moshiri F, Stallings WC, Brown SM *et al.* (2003) The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. *Biochemistry* 42:6696–708

Sanghani SP, Davis WI, Dumaual NG, Mahrenholz A, Bosron WF (2002) Identification of microsomal rat liver carboxylesterases and their activity with retinyl palmitate. *Eur J Biochem* 269:4387–98

Schweizer A, Kornfeld S, Rohrer J (1997) Proper sorting of the cation-dependent mannose 6-phosphate receptor in endosomes depends on a pair of aromatic amino acids in its cytoplasmic tail. *Proc Natl Acad Sci USA* 94:14471–6

Shi YQ, Hubacek I, Rando RR (1993) Kinetic mechanism of lecithin retinol acyl transferase. *Biochemistry* 32:1257–63

Shingleton JL, Skinner MK, Ong DE (1989) Retinol esterification in Sertoli cells by lecithin-retinol acyltransferase. *Biochemistry* 28:9647–53

Siegenthaler G (1990) Retinoic acid formation from retinol and retinal metabolism in epidermal cells. *Methods Enzymol* 189:530–6

Siegenthaler G, Saurat JH, Ponec M (1990) Retinol and retinal metabolism. Relationship to the state of differentiation of cultured human keratinocytes. *Biochem J* 268:371–8

Sincock PM, Ganley IG, Krise JP, Diederichs S, Sivars U, O'Connor B *et al.* (2003) Self-assembly is important for TIP47 function in mannose 6-phosphate receptor transport. *Traffic* 4:18–25

Subramanian V, Garcia A, Sekowski A, Brasaemle DL (2004) Hydrophobic sequences target and anchor perilipin A to lipid droplets. *J Lipid Res* 45:1983–91

Torma H, Vahlquist A (1987) Retinol esterification by mouse epidermal microsomes: evidence for acyl-CoA:retinol acyltransferase activity. *J Invest Dermatol* 88:398–402

Torma H, Vahlquist A (1990) Vitamin A esterification in human epidermis: a relation to keratinocyte differentiation. *J Invest Dermatol* 94:132–8

Tur E, Hohl D, Jetten A, Panizzon R, Frenk E (1995) Modification of late epidermal differentiation in photaged skin treated with topical retinoic acid cream. *Dermatology* 191:124–8

Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, Bickel PE (2005) S3–12, Adipophilin, and TIP47 package lipid in adipocytes. *J Biol Chem* 280:19146–55

Wolins NE, Rubin B, Brasaemle DL (2001) TIP47 associates with lipid droplets. *J Biol Chem* 276:5101–8

Zechner R, Strauss JG, Haemmerle G, Lass A, Zimmermann R (2005) Lipolysis: pathway under construction. *Curr Opin Lipidol* 16:333–40